PCT/GB 0 0 / 0 1 0 7 9

The Patent Office

GB 00/1079

INVESTOR IN PEOPLE

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

I also certify that by virtue of an assignment registered under the Patents Act 1977, the application is now proceeding in the name as substituted.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed R. Mahoney

Dated 6 April 2000

GB9906515.3

By virtue of a direction given under Section of the Patents Act 1977, the application is proceeding in the name of

CANCER RESEARCH VENTURES LIMITED
Cambridge House
6-10 Cambridge Terrace
Regents Park
LONDON
NW1 4JL
United Kingdom

[ADP No. 07822414001]

ts Form 1/77

Patents A[illegible]7
(Rule 16)

The Patent Office

[illegible]99 E434822-1 M2827
[illegible]700 0.00 - 9906515.3

1/77

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office
Cardiff Road
Newport
Gwent NP9 1RH

22 MAR 1999
NEWPORT

1. Your reference — JEC/BP5761911

2. Patent application number *(The Patent Office will fill in this part)* — 22 MAR 1999 **9906515.3**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — EUROPEAN INSTITUTE OF ONCOLOGY
VIA RIPAMONTI 435
20141 MILAN
ITALY

SECTION 30 (1977 ACT) APPLICATION FILED 6/1/2000.

Patents ADP number *(if you know it)* — 76262100[illegible]

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — MATERIALS AND METHODS RELATING TO THE EFFECTS OF P66 EXPRESSION

5. Name of your agent *(if you have one)* — MEWBURN ELLIS

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* — YORK HOUSE
23 KINGSWAY
LONDON
WC2B 6HP

Patents ADP number *(if you know it)* — 109006

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month /year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d)) — YES

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	0
Description	25
Claim(s)	0
Abstract	0
Drawing(s)	11

10. If you are also filing any of the following, state how many against each item

Priority documents	0
Translations of priority documents	0
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	0
Request for preliminary examination and search *(Patents Form 9/77)*	0
Request for substantive examination *(Patents Form 10/77)*	0
Any other documents *(Please specify)*	0

11. I/We request the grant of a patent on the basis of this application.

Signature: Newbury Ellis

Date: 22 March 1999

12. Name and daytime telephone number of person to contact in the United Kingdom: JOANNA CRIPPS 0117 9266411

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed it it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

MATERIALS AND METHODS RELATING TO EFFECTS OF p66 EXPRESSION

Field of the Invention

The present invention relates to materials and methods concerned with the effects of p66 expression. Particularly, but not exclusively, the present invention provides materials and methods relating to observations that p66, and more particularly p66 Shc isoform, is part of a signal transduction pathway that regulates stress response, response to oncogenic signals and lifespan in mammals.

Background of the Invention

The genes that are responsible for the phenomenon of aging in mammals are unknown. Current theories postulate that aging is the consequence of mutations which do not affect fitness of adult individuals, and which have deleterious effects later in life. Circumstantial evidence suggest genes involved in the control of the oxidative stress response are candidate "aging genes". Indeed, accumulation of oxidative damage correlates with aging.

The mammalian SHC locus encodes three isoforms: p52, p46 and p66. They differ by the presence of N-terminal sequences of variable length and share a C-terminal SH2 domain, a central collagen-homology domain (CH1), rich in proline/glycine residues, and an N-terminal phosphotyrosine-binding domain (PTB). The 110 amino acid N-terminal region unique to p66 is also rich in glycine and proline residues (CH2) (Fig.1A). Therefore, $p66^{shc}$ is a splice variant of $p52^{shc}/p46^{shc}$ (Migliaccio E. et al Embo J. 16, 706-716 (1997), a cytoplasmic signal transducer involved in the transmission of mitogenic signals from tyrosine kinases to Ras (Pelicci G. et al Cell 70, 93-104

(1992)). The p52/46 Shc isoforms are involved in the cytoplasmic propagation of mitogenic signals from activated receptors to Ras (Bonfini L. et al Tibs 21, 257-261; 1996). They are rapidly phosphorylated on tyrosine after ligand stimulation of receptors and, upon phosphorylation, form stable complexes with activated receptors and Grb2, an adaptor protein for the Ras guanine nucleotide exchange factor SOS (Migliaccio E. et al Embo J. 16, 706-716 (1997); Pelicci G. et al Cell 70, 93-104 (1992); Rozakis-Adcock, M. et al Nature 360, 689-692 (1992)). These complexes induce Ras activation, as measured by increased RasGTP formation, Mitogen Activated Protein Kinase (MAPK) activity and FOS activation in cultured cells overexpressing $p52^{shc}/p46^{shc}$ (Migliaccio, E. et al Embo J. 16, 706-716 (1997); Pronk, G. et al Mol.Cell. Biol. 14, 1575-1581 (1994); Lanfrancone, L. et al Oncogene 10, 907-917 (1995)). Likewise, $p66^{shc}$ becomes tyrosine-phosphorylated upon receptor activation and forms stable complexes with activated receptors and Grb2. However, it inhibits c-fos promoter activation and does not affect MAPK activity, thereby suggesting that $p66^{shc}$ acts in a distinct intracellular signalling pathway (Migliaccio E. et al Embo J. 16, 706-716 (1997)).

c-fos is transcriptionally activated in response to a large variety of adverse agents (environmental stress), such as DNA-damaging agents (e.g. ultraviolet radiation, UV) or agents that induce oxidative damage (e.g. hydrogen peroxide, H_2O_2) (Schreiber, M. et al Embo J. 14, 5338-5349 (1995); Sen, C. et al FASEB J. 10, 709-720 (1996)).

It is postulated that the major causal factor of aging is the accumulation of oxidative damage as an organism ages (Martin, G. M. et al Nature Genetics 13, 25-34 (1996); Johnson, F.B. et al Cell 96, 291-302 (1999); Lithgow G. J. et al Science 273, 80 (1996)). Indeed, transgenic flies that overexpress antioxidative

enzymes have greater longevity (Orr W. C. et al Science 263, 1128-1130 (1994)); restriction of caloric intake lowers steady state levels of oxidative stress and damage and extends the maximum life span in mammals (Sohal R.S. et al Science 273, 59-63 (1996)). However, the genes that determine lifespan in mammals are not known. Among currently accepted evolutionary theories, it is postulated that aging is a post-reproductive process that has escaped the force of natural selection and that evolved through selection of alleles with early life benefits combined with pleiotropically harmful effects later in life. The postulated genes, since actively selected, are, therefore thought to regulate fundamental cellular processes, common to different species.

Summary of the Invention

The present inventors have determined that p66 is a pivotal gene in the regulation of the cellular responses to environmental and oncogenic stresses and that it is involved in the process of aging and in tumour suppression. p66 provides the first genetic information on the theory of aging. Mechanistically, p66 exerts its functions downstream to stress-activated serine kinases and upstream to p53-p21.

The present inventors have determined that targeted mutation of the mouse $p66^{shc}$ gene induces stress resistance and prolongs survival. The present inventors disclose herein that i) $p66^{shc}$ is serine phosphorylated upon UV treatment or oxidative damage; ii) the serine-phosphorylation of p66 by oxidative signals is mediated by Erk1 and p38, as shown both in vivo and in vitro; iii) ablation of $p66^{shc}$ expression by homologous recombination enhances resistance to oxidative damage both *in vitro* and *in vivo;* iv) a serine-phosphorylation defective mutant of $p66^{shc}$ is unable to restore a normal stress response in

p66^{shc} targeted cells; v) mice carrying the p66^{shc} targeted mutation have prolonged lifespan.

Furthermore, the present inventors have determined that targeted mutation of the mouse p66^{shc} gene increases susceptibility to tumour formation. The present inventors disclose herein that i) p16, p53 and p21 activation is lost in p66-/- cells upon H_2O_2 or UV treatment or RASV12 expression; ii) the oncogenic RASV12 is unable to induce cell senescence into p66-/- MEFs and, on the contrary, it transforms p66-/- cells; iii) p66-/- MEFs over-expressing RASV12 show a transformed, spindle-shaped morphology, are capable of forming foci at confluency and colonies in semisolid media; iv) p16 and p53 are unable to induce growth proliferation of p66-/- cells; v) p66-/- mice are more susceptible to chemically-induced carcinogenesis than littermates.

Thus, the present inventors show herein that p66 itself is activated by serine phosphorylation by stress activated kinases and signals to p16-p19-p53-p21 and that functionally, the p66 signalling pathway regulates tumour supression and lifespan.

Therefore, at its most general, the present invention provides materials and methods associated with the modulation of p66^{shc} gene expression and its involvement in a signal transduction pathway that is activated by environmental stresses and oncogenic mutations.

In a first aspect, the present invention provides a nucleic acid molecule comprising a p66^{shc} coding sequence incorporating at least one mutation as compared to the wild type sequence or the sequence as shown in Fig. 5 such that the protein encoded by the coding sequence has at least one serine residue absent or replaced by a different amino acid residue. Preferably, the serine residue is selected from the group consisting of S17,

S19, S20, S26, S28, S36, S38, S40, S41, S54, S60, S66, S80, S120 and even more preferably selected from the group consisting of S28, S36 and S54. Even more preferably, the serine residue is replaced by a different amino acid residue, for example S36 is replaced by alanine ($p66^{shc}$S36A).

The nucleic acid of the present invention may comprises a $p66^{shc}$ coding sequence which differs further from the wild type sequence or the sequence as shown in Fig. 5 in that it is a nucleic acid sequence that is an allele, mutant, variant or derivative, by way of nucleotide addition, insertion, substitution or deletion of the wild type sequence as illustrated in Fig. 5.

Systematic or random mutagenesis of nucleic acid to make an alteration to the nucleotide sequence may be performed using any technique known to those skilled in the art. In various embodiments of the present invention, a nucleic acid sequence that is a fragment, mutant, allele, derivative or variant, by way of addition, insertion or substitution or more or more nucleotides, of the $p66^{shc}$ wild type sequence as illustrated in Fig. 5, has at least 60% homology, preferably at least 70% homology, more preferably at least 80% homology, more preferably at least 90% homology and even more preferably at least 95% homology.

The present invention further provides a polypeptide encoded for by the nucleic acid molecule of the present invention as disclosed above. A preferred polypeptide comprises the $p66^{shc}$ amino acid sequence or fragment thereof having at least one serine residue present in the wild type $p66^{shc}$ sequence absent or replaced by a different amino acid residue. Preferably, the serine residue is one of S28, S36 or S54 and preferably, it is replaced with an alanine residue (e.g. $p66^{shc}$S36A).

Both the nucleic acid molecules and the polypeptides

as disclosed herein may be used in a method of treatment and in particular may be used in the preparation of a medicament for increasing the cellular resistance to oxidative stress.

Therefore, the present invention also provides methods of increasing resistance in cells to oxidative stress. Such oxidative stress may be as a result of external, e.g. environmental, factors such as UV, X-rays heat shock, osmotic shock, oxidative stress (singlet oxygen, H_2O_2, hydroxylradicals, inflammatory cytokines). or it may be as a result of internal factors resulting in necrosis of cells as occurs in some disease states.

A method of increasing resistance to oxidative stress may comprise disrupting a $p66^{shc}$ signalling pathway. The pathway may be disrupted at any stage during the signalling process, for example, the $p66^{shc}$ polypeptide may be mutated such that the serine residue is absent or replaced by a different amino acid residue, e.g. alanine such that the resulting polypeptide cannot be serine phosphorylated; the ability of molecules such as p38 or MAPK to phosphorylate $p66^{shc}$ may be disrupted by, for example, dominant negative kinases or specific inhibitors; and , most preferably, the expression of $p66^{shc}$ may be disrupted. Further,as p53 and p16 are not biologically active in p66-/- cells, any dominant negative p66 molecules may be used to block p16 and p53 function.

The disruption of $p66^{shc}$ gene expression may be obtained in various ways. Antisense oligonucleotide sequences based on the $p66^{shc}$ sequence may be designed to hybridise to the complementary sequence of nucleic acid, pre-mRNA, or mature mRNA, interfering with the production of polypeptide encoded by a given DNA sequence (e.g. either native $p66^{shc}$ polypeptide or a mutant form thereof), so that its expression is reduced or prevented

altogether. In addition to the $p66^{shc}$ coding sequence, antisense techniques can be used to target the control sequences of the $p66^{shc}$ gene, e.g. in the 5' flanking sequence of the $p66^{shc}$ coding sequence, whereby the antisense oligonucleotides can interfere with the $p66^{shc}$ control sequences. The construction of antisense sequences and their use is described in Peyman and Ulman, Chemical Reviews, 90:543-584, (1990), Crooke, Ann. Rev. Pharmacol. Toxical., 32:329-376, (1992), and Zamecnik and Stephenson, P.N.A.S., 75:280-284, (1974).

In a second aspect, the present invention provides a method of screening for compounds capable of modulating a $p66^{shc}$ signalling pathway comprising contacting a candidate compound with a $p66^{shc}$ expression system; determining the amount of a component of the signalling pathway; and comparing said amount of the component with the amount of the component in the absence of said candidate compound.

Preferably, the expression system comprises a nucleic acid vector having a $p66^{shc}$ coding sequence or fragment thereof inserted therein. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, termination fragments, polyadenylation sequences enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, Molecular cloning: A laboratory Manual: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press. Procedures for introducing nucleic acid into cells depends on the host cell used, but are well known.

Thus the expression system may also comprises a host cell containing a $p66^{shc}$ coding sequence or a vector as disclosed above. Most preferably, the expression system comprises a cell derived from a cell line, such as mouse embryo fibroblasts, known to express $p66^{shc}$.

The $p66^{shc}$ signalling pathway may be modulated, e.g. disrupted, at any stage during the signalling pathway such that production of active $p66^{shc}$ is prevented. Examples of such modulation may include directly preventing expression of $p66^{shc}$ by blocking factors involved in the transcription of genes. For example, antisense primers may be used to bind to the $p66^{shc}$ gene thereby preventing transcription factors binding to regulatory agents required for promoting transcription. Alternatively, the coding sequence for $p66^{shc}$ may be targeted so as to introduce mutations which prevent the expression of $p66^{shc}$ without disrupting expression of associated proteins such as $P52^{shc}$ and $p46^{shc}$. Preferably, the mutation disrupts the exon encoding the p66 CH2 region. Antisense probes may also be used for binding DNA or mRNA encoding $p66^{shc}$ such that its translation is prevented. Alternatively, antibodies specific for $p66^{shc}$ (e.g. anti-CH2 antibodies) may be used to specifically bind to the expressed protein such that subsequent binding of $p66^{shc}$ to other proteins, e.g. receptors, is prevented.

Further, inhibition of the p66 function can be obtained by inhibiting the phosphorylation of the p66 CH2 region induced by Erk 1 or p38 stress-kinases. To this end, library of compounds can be screened to identify those which are able to inhibit the in vitro phophorylation of the GST-CH2 region by recombinant Erk1.

Thus, the present invention provides use of $p66^{shc}$ for screening for molecules which affect or modulate its activity or function. Such molecules may be useful in a therapeutic (possibly including prophylactic) context.

It is well known that pharmaceutical research leading to the identification of a new drug may involve screening of very large numbers of candidate substances, both before and even after a lead compound has been

found. This is one factor which makes pharmaceutical research very expensive and time consuming. Means for assisting in the screening process can have considerable commercial importance and utility. Such means for screening for substances potentially useful in increasing resistance to oxidative stress and thus extending cellular longevity, is provided by the present invention.

A method for screening for a substance which modulates (disrupts) activity of the $p66^{shc}$ polypeptide may include contacting one or more test substances with the polypeptide in a suitable reaction medium, testing the activity of the treated polypeptide and comparing that activity with the activity of the polypeptide in comparable reaction medium untreated with the test substance or substances.

Combinatorial library technology provides an efficient way of testing a potentially large number of different substances for ability to disrupt or delete activity of $p66^{shc}$. Such libraries and their use are known in the art. The use of peptide libraries is preferred.

Further, assays for determining p66 inhibitors may include usage of wildtype cells (both established cell lines or primary cells capable of expressing $p66^{shc}$, e.g. MEFs, p66-/- MEFS or MEFs overexpressing p66 (through the usage of a $p66^{shc}$ expressing vector).

Comparative responses to be determined may include response to stress factors, e.g. UV or H_2O_2; inhibition of RASV12 (or any other oncogene) -induced senescence in primary fibroblasts; inhibition of p53 or p19 or p16 or p21 function (as measured by transcriptional assays, or stability assays, or nucleus-cytoplasmic export assays); or inhibition of p66 phosphorylation induced by RASV12 or by oxidative stress signals.

Following identification of a substance or compound which disrupts $p66^{shc}$ or a step in the $p66^{shc}$ signalling

pathway, the substance or compound may be investigated further. Furthermore, it may be manufactured and/or used in the preparation, i.e manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

The present invention also provides a method of increasing cellular resistance to oxidative stress comprising deleting or disrupting the gene encoding $p66^{shc}$ from the cellular genome. Such a method may include processes such as gene therapy wherein a nucleic acid vector comprises a nucleic acid sequence capable of being incorporated into the genome of the cell and disrupting the expression of $p66^{shc}$.

The present invention further provides genetic markers for aging. Such markers may provided materials and methods for determining a predisposition to aging associated with certain disease states. As disclosed herein, heterozygous p66+/- mice have a slight, but evident, increase in longevity, thereby suggesting that slight variations in the expression levels of p66 may influence aging. This may provide for the determination of variations in the p66 RNA transcriptional regulatory sequences (e.g. the promoter) which affect the p66 transcription rate and longevity. Also, since the lifespan correlates with the functional activity of p66, there may be allelic variations in the p66 coding sequence that also correlate with different length of lifespan.

In a further aspect of the present invention, there is provided methods of increasing resistance to tumour formation by increasing expression of $P66^{shc}$. The results disclosed herein indicate that increased levels of $P66^{shc}$ reduce susceptibility to carcinogenesis. Therefore, the present invention further includes the use of $P66^{shc}$

(nucleic acid molecules or polypeptides as disclosed herein) for reducing susceptibility to cancers. Likewise, the present invention provides use of $P66^{shc}$ in the preparation of a medicament for the treatment and prevention of cancers.

$P66^{shc}$ for such use, may be in the form of a polypeptide or may be in the form of a nucleic acid molecule which encodes a functional $P66^{shc}$ polypeptide. The nucleic acid may be in the form of an expression vector which comprises a nucleic acid molecule encoding part or all of $P66^{shc}$ polypeptide. The expression vector may be used as part of a gene therapy application as disclosed herein. Further, in this aspect, agents may be used which increase the expression of $P66^{shc}$ within the cell. Such agents may be further nucleic acid molecules or compounds such as transcription factors, which are capable of increasing the expression of $P66^{shc}$ or compounds that increase the levels of p66 expression acting at post-transcriptional levels, such as at the level of stability of RNA or protein.

Aspects and embodiments of the invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects of the invention will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

Brief Description of the Drawings

In the figures

Figure 1 shows serine phosphoryation of $p66^{shc}$ by UV or H_2O_2 treatment. Figure 1A: Modular organisation of $p66^{shc}$; Y: Y239, Y340 and Y317, the major Shc tyrosine phosphoryation sites. The alternative initiation codon (ATG) of $p46^{shc}$ is indicated. Figure 1B: Anti-phosphotyrosine Western blotting of anti-p66 (αCH2)

immunoprecipitates from lystates of serum starved MEFs (SF) or Mouse Embryo Fibroblasts (MEFs) treated with EGF, UV or H_2O_2 for 5 min or 4 hrs, as indicated. The same blot was reprobed with anti-$p66^{shc}$ antibodies (α-CH2). The $p66^{shc}$ polypeptides are arrowed. Immunoglobulin cross-reactive polypeptides are also indicated (Ig). Figure 1C: Western blotting analysis of $p66^{shc}$ expression of serum starved MEFs (SF) or MEFs treated with EGF, UV or H_2O_2 for 5 min or 4 hrs as indicated. The same blot was reprobed with anti-actin antibodies. Figure 1D: Phosphoaminoacid analysis of $p66^{shc}$. Serum-starved MEFs (SF) were labelled with 1mCi/ml [^{32}P] orthophosphate for 4 hr and unstimulated (SF) or cells stimulated with EGF, UV or H_2O_2 for 5 min or 4 hr were lysed and immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose and autoradiographed (not shown). Phosphoaminoacid analysis was performed on the $p66^{shc}$ polypeptide. Positions of the phosphoserine (S), phosphothreonine (T) and phosphotryosine (Y) markers are indicated.

Figure 2 shows $p66^{shc}$ enhances stress oxidative response *in vitro* and *in vivo*. Figure 2A: Western blotting analysis of Shc expression in $p66^{shc}$+/+ or $p66^{shc}$-/- MEFs (left panel) and in $p66^{shc}$-/- MEFs transduced with vector alone, $p66^{shc}$ or $p66^{shc}$S36A cDNAs (right panel). Figure 2B: Viability of MEFs after H_2O_2 treatment. Equal numbers (1.2 x 15^5) of the indicated MEF cells grown in 100mm dishes in triplicate were infected with the PINCO retrovirus or with PINCO retroviruses expressing $p66^{shc}$ or $p66^{shc}$S36A, as indicated, kept for additional 48 hrs to allow gene expression of exogenous cDNAs and exposed to 400mM H_2O_2 for 24 hrs. Cell viability was determined by trypan blue exclusion. Results are expressed as a percentage of viable cells with respect to H_2O_2 untreated controls and represent the mean of three independent experiments. Expression of $p66^{shc}$ or $p66^{shc}$S36A did not

significantly influence viability or growth rate of MEFs, as measured 48 hrs after viral infection (not shown).

Figure 3 shows mapping of $p66^{shc}$ serine-phosphorylation sites. Figure 3A: Anti-CH2 western blots of lystates from MEFs transfected with vectors expressing the isolated CH2 region and then starved (SF) or treated with EGF or UV for 5 min or 4 hr, as indicated. The star indicates the shifted CH2 polypeptide. Figure 3B: The S36A or S54A mutations were introduced with the isolated CH2 region or the full-length $p66^{shc}$. The resulting cDNAs were HA-tagged, cloned with a pcDNA3 expression vector and transfected into MEFs. Cultures were treated as indicated and analysed by western blotting using anti-HA antibodies. Figure 3C: Phosphoaminoacid analysis of $p66^{shc}$ and $p66^{shc}$S36A. MEFs were transfected with HA-$p66^{shc}$ or HA $p66^{shc}$S36A expression vectors, kept in culture for 48 hrs labelled with 1mCi/ml[^{32}P] orthophosphate for 4 hr and treated with EGF or H_2O_2 for 5 min, as indicated, lysed and immunoprecipitated with anti-HA antibodies. Phosphoaminoacid analysis was performed on the HA-$p66^{shc}$ or HA-$p66^{shc}$S36A polypeptides, as described in Fig. 1 legend.

Figure 4 shows cumulative survival (Kaplan and Meier) or $p66^{shc}$+/+ (dashed line), $p66^{shc}$+/- (dotted line) and $p66^{shc}$-/- (solid line) mice. Survival of the $p66^{shc}$-/- mice was 71.4%.

Figure 5 shows the p66 cDNA nucleotide sequence (the ATG initiation site is underlined) and, separated, the p66 amino acid sequence.

Figure 6 shows a 13kb genomic region containing all the Shc coding exons which was characterised by restriction enzyme mapping and nucleotide sequence of all exon-intron boundaries and 5' regulatory regions.

Figure 7 shows the construction of the targeting

vector pBSp66ShcKO.

Figure 8 shows the vector pBSp66ShcKO with a TK transcriptional unit cloned at its 3' end.

Detailed Description

Methods

1) Cell lines, reagents and plasmid construction

Mouse embryo fibroblasts (MEFs) were isolated from 12 to 14 day embryos derived from $p66^{shc}$-/- mice and $p66^{shc}$+/+ mice and maintained in Earle's minimal essential medium supplemented with 10% fetal bovine serum. The S36A and S54A mutations were generated by standard PCR techniques. The $p66^{shc}$, $p66^{shc}$S36A, HA-CH2, HA-CH2S35A, HA-CH2S54A, HA-$p66^{shc}$, HA-$p66^{shc}$-S35A and HA-$p66^{shc}$S54A were cloned into the pCDNA3 or PINCO eukaryotic expression vectors (Claudio P.P. et al Cancer Res. 54, 5556-5560 (1994); Grignani, F. et al Cancer Res. 1, 14-19 (1998)). The antibodies used were: the anti-Shc polyclonal antibody which recognises the SH2 domain of all three Shc isoforms (Pelicci G. et al Cell 70, 93-104 (1992)); the anti p66 polyclonal antibody which recognises the $p66^{shc}$ isoform (Migliaccio E. et al Embo J. 16, 706-716 (1997)); the anti-βactin polyclonal antibody, (Sigma Immuno Chemicals); the anti-HA monoclonal antibody; the anti-phosphotyrosine monoclonal antibody, (Santa Cruz Biotechnology).

2) Metabolic labeling immunoprecipitaion, Western blotting and phosphoaminoacid analysis.

For whole lysates, cells were directly lysed in SDS sample buffer (50mM Tris-HCL pH 6.8, 2% SDS v/v, 10% glycerol and 5% v/v β-mercaptoethanol) and boiled for 5

min. 50µg of total protein was analysed by SDS-PAGE. For immunoprecipitation, cells were lysed on ice in PY buffer (20mM Tris-HCL ph 7.8, 50mM NaCl, 30mM $Na_4P_2O_7$. 5mM sodium orthovanadate, 1% v/v Triton x-100 containing freshly added protease inhibitors: 1mM phenylmethyl sulfonhyl fluoride, 10µgml^{-1} leupeptin and 5 mg ml^{-1} aprotinin), appropriate antibodies were adsorbed on Protein A Sepharose (Pharmacia) and then incubated with cell lysates for 2hr at 4°C. Immunoprecipiates were recovered, resolved by 10% SDS-PAGE and transferred to nitrocellulose filters, as described elsewhere (Migliaccio E. et al Embo J. 16, 706-716 (1997)). Blots were blocked, probed with specific antibodies and immune complexes revealed by horseradish-peroxidase conjugated with specific secondary antiserum (Biorad) followed by enhanced chemiluminescence. For phosphoaminoacid analysis, cells were grown to confluence on 10 cm plates, starved in serum-free medium and labelled for 4h in 5 ml phosphate free DMEM containing 5% dialyzed FBS and 1mCi ml^{-1} ^{32}P-orthophosphate. Cells were stimulated with 30ng ml^{-1} EGF or 400µM H_2O_2, or irradiated with 50 J/m^2 UV, rinsed twice with ice cold PBS and lysed in PY buffer. $p66^{shc}$ proteins were isolated by immunoprecipitation with anti-SHC or anti-HA antibodies and resolved by SDS-PAGE. $p66^{shc}$ polypeptides were transferred to PVDF membranes and hydrolyzed in 6M HCl for 60 min at 110°C. The hydrolysis products were separated in the presence of phosphoserine, phosphothreonine and phosphotyrosine markers by SDS-PAGE at pH1.9 and pH3.5 in two dimensions on TLC plates.

3) Transfections, infections and cellular viability test.

MEFs were transfected with the LipofectaMINE PLUS Reagent GibcoBRL (average transfection efficiency 50%). For retroviral infections, the empty PINCO vector and recombinant PINCO vectors expressing $p66^{shc}$ or $p66^{shc}$S36A

cDNAs were transfected into the phoenix amphotropic packaging cell line and, after 48 hrs, supernatants were used to infect MEFs cells (1.2 $x10^5$ cells/100mm dish). The efficiency of infection (GFP positive cells) was determined by FACS analysis 48 hr after infection. Viability was assessed by the trypan-blue dye exclusion test.

4) Statistical analysis

Survival functions were estimated by the Kaplan and Meier product limit method. Survival distributions were compared using the logrank test (Marubini E. et al New York, John Wiley & Sons (1995). All statistical calculations were performed using SAS/STAT Rel. 6.12 software (SAS Institute 1995).

5) Construction of $p66^{shc}$ targeting vector and electroporation and selection of ES cells.

The targeting vector was constructed using standard cloning procedures. The plasmid was linearized with Kpn 1 before electroporation into ES cells. CJ7 ES cells were maintained on a monolayer of mitomycyn C inactivated, neomycin-resistant primary embryonic fibroblast. A suspension of 15 million trypsinized ES cells in PBS was electroporated with 25 µg of DNA of the linearized targeting vector by using the Bio-Rad gene pulser II apparatus with 240 V and 500 µF. Cells were plated immediately after transfection and allowed to recover for 24 hr before selection in medium 350 µg/ml Geneticin and 2 µM of ganciclovir. Cells were fed daily and after 9 days the resulting colonies were picked and cultivated singularly until extraction and freezing.

6) Southern blot analysis of ES cells and Mice.

To identify the mutated Shc allele, genomic DNA from

ES cells and from the mouse tails was prepared by proteinase K Iysis and phenol-chloroform extraction, digested with Eco R1 and analyzed by Southern blot analysis. A 1.1.Kb Eco R1-Xba 1 fragment was used as external probe to discriminate between the WT 8 kb and the recombinant 3.5 kb allele bands (Fig 2).

7) Generation of mice carrying the disrupted p66 Shc allele.

Two different clones of targeted ES were used to generate chimeric mice. C57BL/6J blastocysts injected with 10-15 ES cells were transferred to pseudopregnant female mice. Chimeric mice, identifiable by agouti coat color, were mated with C57BL/6J mice. Offspring with agouti coat color were tested for the presence of the recombined allele by means of Southern blot analysis. Heterozygotes obtained from the crosses of the chimeras with 129Sv female mice were interbred to establish the colony of p66 -/- mice in the 129 genetic background. The mice were housed at a constant room temperature (22°C) and humidity (60%) with a 12 h light/dark cycle, with free access to standard mouse chow and tap water.

Results

1) Construction of the p66shc targeting vector.

The present inventors have mutated the mouse Shc locus using conventional embryonic stem cell technology. The genomic Shc locus was isolated from a 129 mouse genomic library. A 13 Kb genomic region containing all the Shc coding exons was characterized by restriction enzyme mapping and nucleotide sequence of all exon-intron boundaries and 5' regulatory regions (Fig. 6). A positive/negative (G418/Ganciclovir) selection strategy was applied to introduce a mutation in the region of

first coding exon that contains the p66 ATG. To construct the targeting vector (pBSp66ShcKO; Fig 7) we used the EcoR1 8 KB fragment containing exons 1-8 (Fig.7). The Bal 1 fragment containing the p66 specific start site (Fig.7) was substituted with the Neo transcriptional unit driven by the pY promoter. The resulting vectors contains 2,2 and 4,6 Kb flanking sequences at the 5' and 3' ends, respectively (Fig. 7). A TK transcriptional unit was cloned at its 3' end (Fig.3).

2) ES tranfection and selection.

CJ7 ES cells, provided by Dr, Vera Soares (Memorial Sloan Kettering Cancer Center, NY, USA) were transfected by electroporation, and resistant colonies were selected and screened for the deletion of the p66 first coding sequence by Southern blotting. The screening strategy was based on the EcoR1 site introduced in the targeted allele by the insertion of the Neo transcriptional unit (Fig.7) 24 ES clones of 150 analyzed showed one targeted Shc allele. Cytogenetic analysis of 9 ES clones revealed a normal modal number of chromosomes.

3) Generation of targeted mice.

Two ES clones were injected into C57BL/6J blastocysts, according to established procedures. Breeding of heterozygous ($p66^{sch}$+/-) yielded the expected frequency of homozygous animals. Analysis of the genotype of the animals was performed by Southern blotting of DNA extracted from the tails and confirmed by Western blotting of $p66^{shc}$ expression in various tissues.

4) p66 is phosphorylated on Ser36 after H_2O_2 or UV stimulation.

Since Fos is transcritionally activated by a variety of environmental stresses (hydrogen peroxide: H_2O_2; ultraviolet irradiation: UV), the present inventors have analysed the modifications of p66 upon H_2O_2 or UV stimulation of mouse and human fibroblasts. Results revealed that p66 is markedly phosphoryated on serine upon H_2O_2/UV irradiation. Further, the present inventors mapped the major serine phosphoryation site to Ser 36. A Ser-Ala 36 p66 mutant is not phosphorylated by UV/H_2O_2 in vivo.

5) Erk1 and p38 mediates UV/H_2O_2 phosphorylation of p66

By using purified enzymes in *in vitro* kinase assays and either dominant negative kinases or specific inhibitors *in vivo*, the present inventors have demonstrated that p66 is phosphorylated by p38 and Erk1 upon UV or H_2O_2 stimulation.

Erk 1 (also known as MAPK), JNK and p38 are stress-induced kinases. To identify the kinase(s) responsible for the phosphorylation of p66 induced by oxidative stess in vivo, the present inventors analysed the extent of p66 phosphorylation after stress signals in MEFs expressing a JNK dominant negative kinase or in cells treated with various MAPK and p38 specific inhibitors [PD98059, which prevents activation of Erk 1 by Raf; SB203580 which specifically inhibits the p38 Map kinases]. Results showed that SB203580 and PD98059, but not the JNK dominant negative kinase, prevented p66 phosphorylation by oxidative stress signals (H_2O_2), indicating that p66 is phosphorylated in vivo by Erk1 and p38, but not by JNK.

The present inventors then reconstructed the p66 phosphorylation in vitro by using recombinant Erk1 or JNK and the bacterially expressed p66 CH2 region (CH2 was expressed in bacteria as GST-fusion protein). Erk1, but not JNK, was unable to in vitro phosphorylate the p66 CH2

region. Phosphorylation was specific, as shown by the finding that, in the same assay, Erk1 was unable to phosphorylate the p66 CH2 region when the S36A mutation was introduced.

6) p66 modulates the oxidative stress response *in vitro*

H_2O_2 treatment induces fibroblast cell death. The present inventors have demonstrated that: i) overexpression of p66 in wild-type MEFs increases cell death induced by H_2O_2; ii) p66-/- MEFs are more resistant to H_2O_2-induced cell death than wild-type controls *in vivo*. Paraquat is a pesticide that kills mice by inducing oxidative damage. The present inventors have further demonstrated that p66-/- mice are more resistant to paraquat treatment than littermates.

7) p66 regulates the p16, p53 and p21 response

Since environmental stresses activates the p16 - p53-p21 signalling pathways, the present inventors have further investigated whether p66 interferes with p16-p53-p21 activation by H_2O_2. Results revealed that p16, p53 and p21 activation are lost in p66-/- cell upon H_2O_2 treatment.

8) p66 is a tumour supressor

In vitro, the stimulatory effect of p66 on the p53-p21 pathway suggests that it might play a role in the cellular response on oncogenic stimuli. Therefore, the present inventors have evaluated the effects of p66 on the response of primary fibroblasts on the oncogenic RASV12 mutant. RASV12 induces senescence of wild-type MEFs, as a consequence of p53-p21 activation. Expression of RASV12 into p66-/- MEFs induced cellular transformation. *In vivo*, p66-/- mice are more susceptible

to chemical-induced carcinogenesis than littermates. Furthermore, the present inventors have demonstrated that p53 and p16 are unable to induce senescence of mouse p66-/- fibroblasts.

9) p66 mediates aging

The results presented herein demonstrate that p66 is involved in the cellular response to stresses (environmental and oncogenic). The present inventors therefore considered whether p66 is also involved in mediating aging. To do this, they evaluated the survival of p66-/- mice. Of the many mice that were born in August 1996, 14+/+, 8+/- and 15-/- were not sacrificed and kept for survival analysis. Evaluation after 28 months (December 1998) revealed

+/+	0/14 survivors	0%
+/-	3/8 survivors	37%
-/-	11/15 survivors	73%

Discussion

To investigate its role in the cellular stress response, the present inventors analysed the extent of $p66^{shc}$ tyrosine-phosphorylation in mouse embryo fibroblasts (MEFs) treated with UV or H_2O_2, as compared to the effects of treatment with growth factors, such as the epidermal-growth factor (EGF). Anti-p66 immunoprecipitates from lysates of untreated and EGF-, UV-, or H_2O_2-stimulated fibroblasts were immunoblotted with anti-phosphotyrosine antibodies. EGF stimulation induced a marked increase in the phosphotyrosine content of $p66^{shc}$, which was maximal after 5 min. Neither UV nor H_2O_2 treatment induced significant tyrosinephosphorylation of $p66^{shc}$ (Fig.1B). However, Western blotting of the same lysates with anti-Shc antibodies revealed a marked gel

retardation of the $p66^{shc}$ polypeptides after 5 min and 4 hours treatment with UV or H_2O_2, consistent with other post-translational modifications of $p66^{shc}$ induced by these agents (Fig.1C). Therefore, $p66^{shc}$ phosphorylation was analyzed by phosphoaminoacid analysis (Fig.2C). $p66^{shc}$ polypeptides from serum-starved cells were phosphorylated primarily on serine. UV (Fig.2C) and H_2O_2 (not shown) induced a marked increase in the level of phosphoserine and had no effect on phosphotyrosine. In contrast, EGF induced a marked increase in the level of phosphotyrosine and a modest increase in phosphoserine (Fig.2C). It appears, therefore, that $p66^{shc}$ is involved in the intracellular transduction pathways of both environmental stresses and growth factors, albeit with distinct functions, since UV and H_2O_2 induced rapid and persistent serine-phosphorylation, while EGF induced rapid and transient tyrosine-phosphorylation.

To investigate the functional role of $p66^{shc}$ in the stress oxidative response, the present inventors next analysed the effects of $p66^{shc}$ overexpression or $p66^{shc}$ ablation on the cellular response of MEFs to H_2O_2. MEFs were derived from mice carrying a targeted mutation of the Shc locus that disrupted the exon encoding the p66 CH2 region, without affecting the $p52^{shc}/p46^{shc}$ coding sequences (M. Giogio et al : submitted for publication). $p66^{shc}+/+$ MEFs from wild-type mice with otherwise identical genetic background were used for comparison. As expected, expression of $p66^{shc}$ was normal in $p66^{shc}+/+$ MEFs while was undetectable in $p66^{shc}-/-$ MEFs; expression $p52^{shc}/p46^{shc}$ was identical in $p66^{shc}+/+$ and $p66^{shc}-/-$ MEFs. $p66^{shc}+/+$ MEFs were susceptible to H_2O_2 treatment, with more than 70% of cells being killed after 24 hours exposure to 400 µM H_2O_2. Overexpression of $p66^{shc}$ rendered $p66^{shc}+/+$ more susceptible to H_2O_2 treatment (approximately 85-90% cell death after 24 hours). In contrast, $p66^{shc}-/-$

MEF cells were more resistant to killing by the same dose of H_2O_2 and more than 70% of these cells survived after 24 hours of H_2O_2 treatment (Fig.2B). Expression of the $p66^{shc}$ cDNA into $p66^{shc}$-/- cells restored a normal response to H_2O_2 (Fig.2B).

The present inventors then examined the ability of $p66^{shc}$-/- mice to resist oxidative stress *in vivo*. To this end, mice were treated with paraquat, which, upon intake by the cell, generates superoxide anion. At a dosage of 70mg/kg, 5 of 5 $p66^{shc}$+/+ mice died within 48 hours after paraquat administration. In contrast, out of 5 $p66^{shc}$-/- treated mice, two died within the first 48 hours, two after approximately 72 hours and 1 survived for several weeks (Fig.2C). Together, these results point to a function of $p66^{shc}$ in the cellular stress oxidative response.

To investigate whether $p66^{shc}$ participates in the cellular stress response as a cytoplasmic transducer of stress signals, The present inventors analysed the potential of a serine-phosphorylation defective mutant of $p66^{shc}$ to rescue the impaired stress oxidative response of $p66^{shc}$-/- MEFs. The $p66^{shc}$ CH2 region probably contains the $p66^{shc}$ major serine-phosphorylation site(s), as suggested by the gel-mobility shift induced by H_2O_2 and EGF, when the CH2 was expressed in cultured cells as isolated domain (Fig.3A). The CH2 region contains three serine residues with a consensus sequence for serine/threonine kinase phosphorylation (S28, S36 and S54) (Davis, J. J. Biol. Chem. 268, 14553-14556 (1993)). Alanine substitution of S36 abrogated the gel-mobility shift of both isolated CH2 and full-length $p66^{shc}$ induced by H_2O_2 (Fig.3B). Phosphoaminoacid analysis of the $p66^{shc}$ and $p66^{shc}$S36A polypeptides revealed a marked increase in the level of phosphoserine induced by H_2O_2 in the $p66^{shc}$, but not in the $p66^{shc}$S36A mutant (Fig.3C), thereby confirming

that S36 is the $p66^{shc}$ major serine phosphorylation site.

The present inventors then expressed the $p66^{shc}$S36A mutant into $p66^{shc}$-/- MEFs and evaluated its effect on the stress oxidative response. As shown in Fig. 2B, $p66^{shc}$S36A was unable to restore a normal response to H_2O_2. Instead, it conferred further resistance to H_2O_2-induced cell death, probably through a dominant negative effect on the stress response-signalling pathway. Together, these results indicate that $p66^{shc}$ acts as a signal transducer in the cellular response to oxidative stress.

Enhanced resistance to environmental stress correlates with prolonged lifespan in invertebrates. In *S.Cerevisiae,* deletions of RAS1 (Sun, J. et al J. Biol. Chem. 269, 18638-18645 (1994); Kale, S. P. et al Dev. Genet. 18, 154-160 (1996)) or mutations in the SIR4 (Kennedy, B. K. et al Cell 80, 485-496 (1995)) locus increase lifespan and resistance to starvation, ethanol and heat shock or UV, respectively. In C. *elegans,* mutants of genes of the dauer signalling pathway, such as age-1 and daf-2, survive longer and are more resistant to oxygen radicals, heat and UV (Murakami, S. et al Genetics, 143, 1207-1218 (1985); Larsen, P. L. et al Genetics 139, 1576-1583 (1995)). In *D.melanogaster,* selection for late-life fitness is associated with greater resistance to environmental stresses (Service, P. M. et al Physiol, Zool. 58, 380-389 (1985); Service P.M. Physiol Zool. 60, 321-326 (1987); Arking R. et al Dev. Genet. 12 362-370 (1991)), and hypomorphic mutants of the *mth* locus live 35% longer and are more resistant to dietary paraquat and starvation (Lin, Y. J. et al Science 282, (1998). The present inventors, therefore, retrospectively analysed the effects of the $p66^{shc}$ mutation on lifespan. 37 mice born on August 1996 from $p66^{shc}$+/-heterozygous parents were not sacrificed and maintained under identical conditions of stability. They

consisted of 14 $p66^{shc}+/+$, 8 $p66^{shc}+/-$ and 15 $p66^{shc}-/-$ mice. After 28 months of observation, all the wild-type animals had died (median survival of 25.37±0.63 months), while 3 of the 8 heterozygous (37%) and 11 of the 15 homozygous (73%) were still alive. The remaining 3 $p66^{shc}+/-$ died after additional two months (median survival of 27.40±2.819). 3 $p66^{shc}-/-$ mice also died after two months; the remaining 9 are still alive (lifespan more than 31 months). The comparison of survival curves obtained by the Kaplan and Meier method (Marubinii, E. et al Valsecchi, M.G. New York, John Wiley & Sons (1995))(Fig.4) showed a highly significant difference between the three groups (log-rank p=0.0002). Cumulative survival did not differ significantly between wild type and heterozygous (p=ns [0.057]). The cumulative survival in the $p66^{shc}+/+$ group was 71.4% ($p<0.01$ vs $p66^{shc}+/-$ and $p66^{shc}+/+$). Therefore, it appears that homozygous mutation of $p66^{shc}$ correlates with prolonged survival in mice.

The results presented herein are consistent with a model in which lifespan is determined as a result of the increased ability to resist or repair environmental damage. $p66^{shc}$ is part of a signal transduction pathway, which is activated by environmental stresses (H_2O_2 or UV) and whose mutation increases stress resistance and lifespan. Biochemical and genetic investigation of the $p66^{shc}$ signalling pathway should lead to better understanding of mechanisms relevant to aging in mammals.

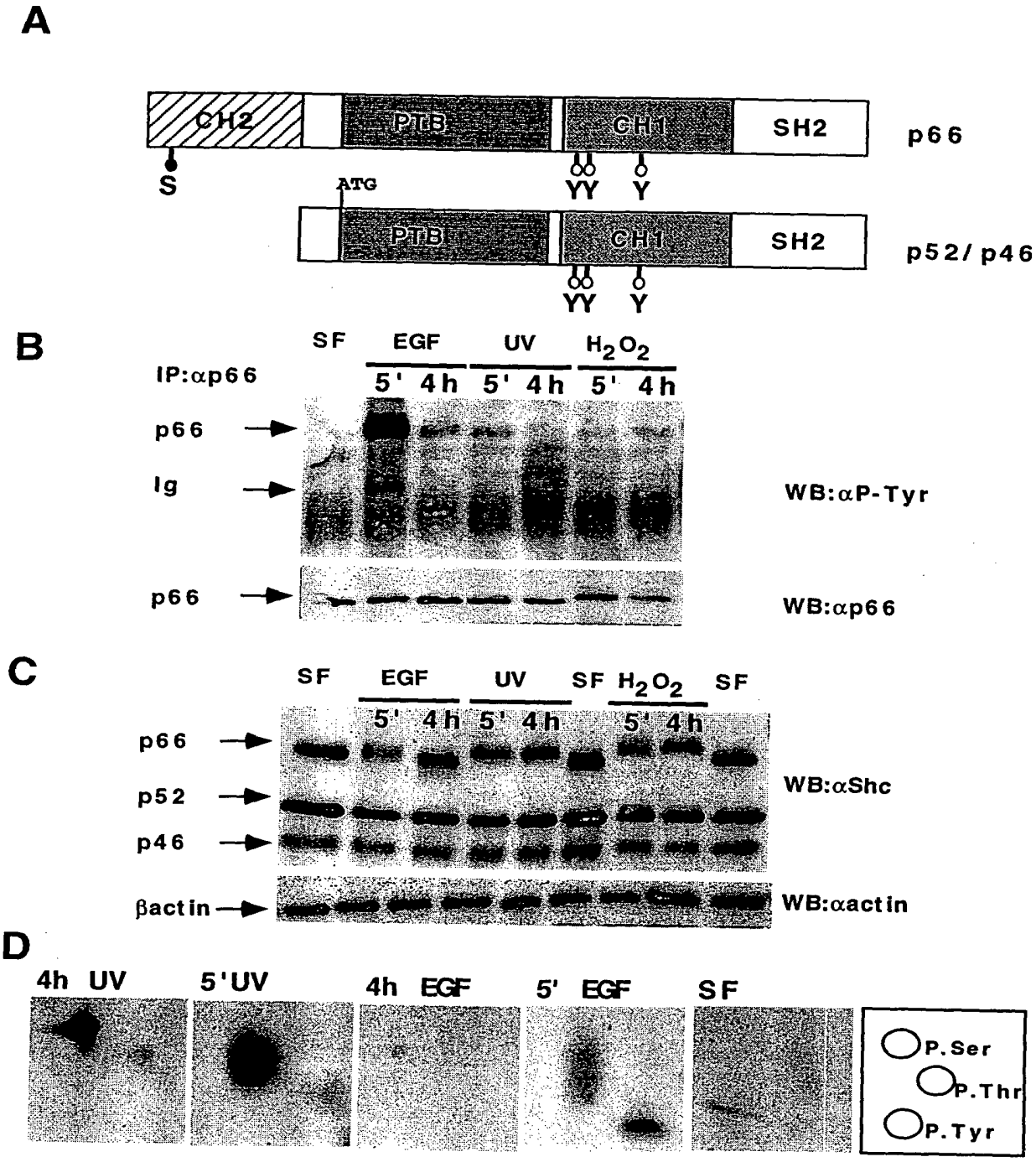
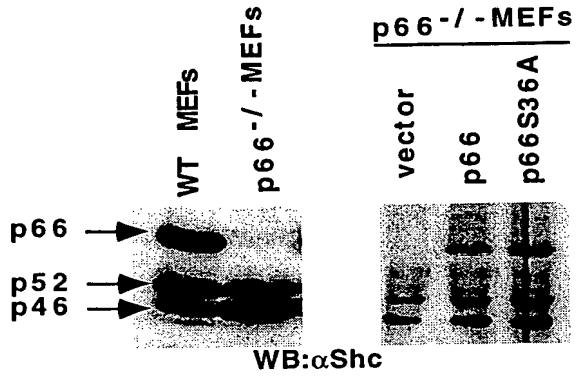
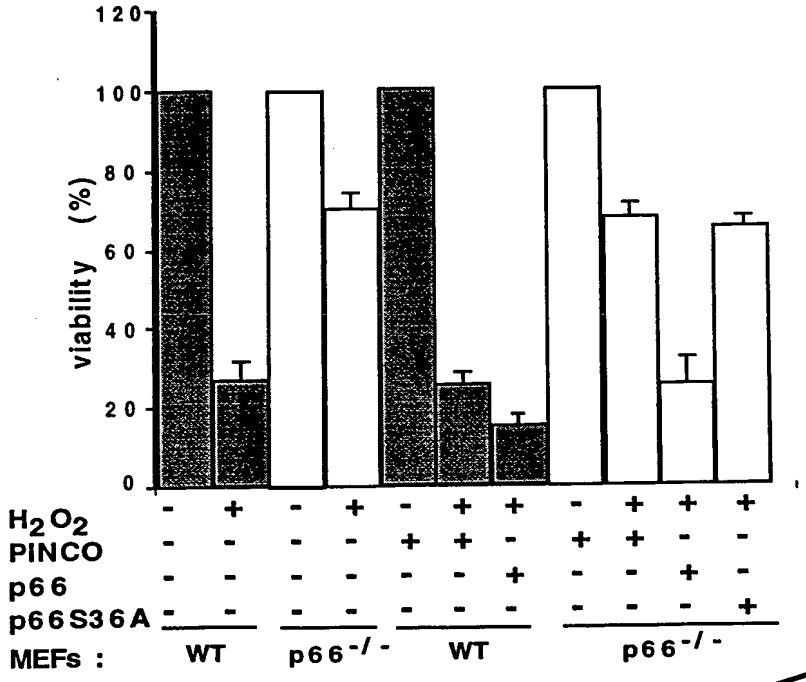

A
CH2
PTB
CH1
SH2
p66
S
ATG
YY
Y
PTB
CH1
SH2
p52/p46
YY
Y
B
SF
EGF
UV
H2O2
5' 4h 5' 4h 5' 4h
IP:αp66
p66
Ig
WB:αP-Tyr
p66
WB:αp66
C
SF
EGF
UV
SF
H2O2
SF
5' 4h 5' 4h 5' 4h
p66
p52
p46
WB:αShc
βactin
WB:αactin
D
4h UV
5'UV
4h EGF
5' EGF
SF
P.Ser
P.Thr
P.Tyr

Fig. 1

A


WT MEFs
p66-/- MEFs
p66-/- MEFs
vector
p66
p66S36A
p66
p52
p46
WB:αShc

B


viability (%)
120
100
80
60
40
20
0
H2O2
PINCO
p66
p66S36A
MEFs :
WT
p66-/-
WT
p66-/-

C

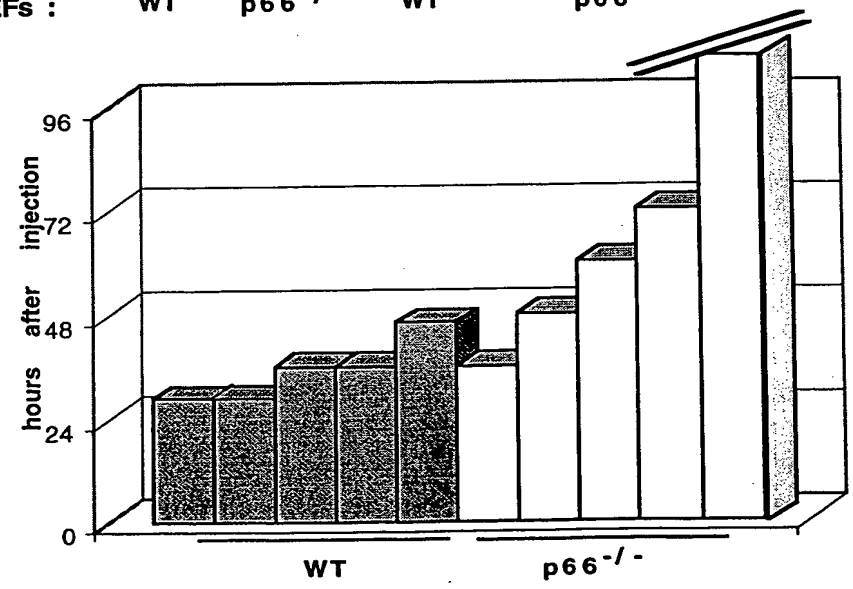

hours after injection
96
72
48
24
0
WT
p66-/-

Fig. 2

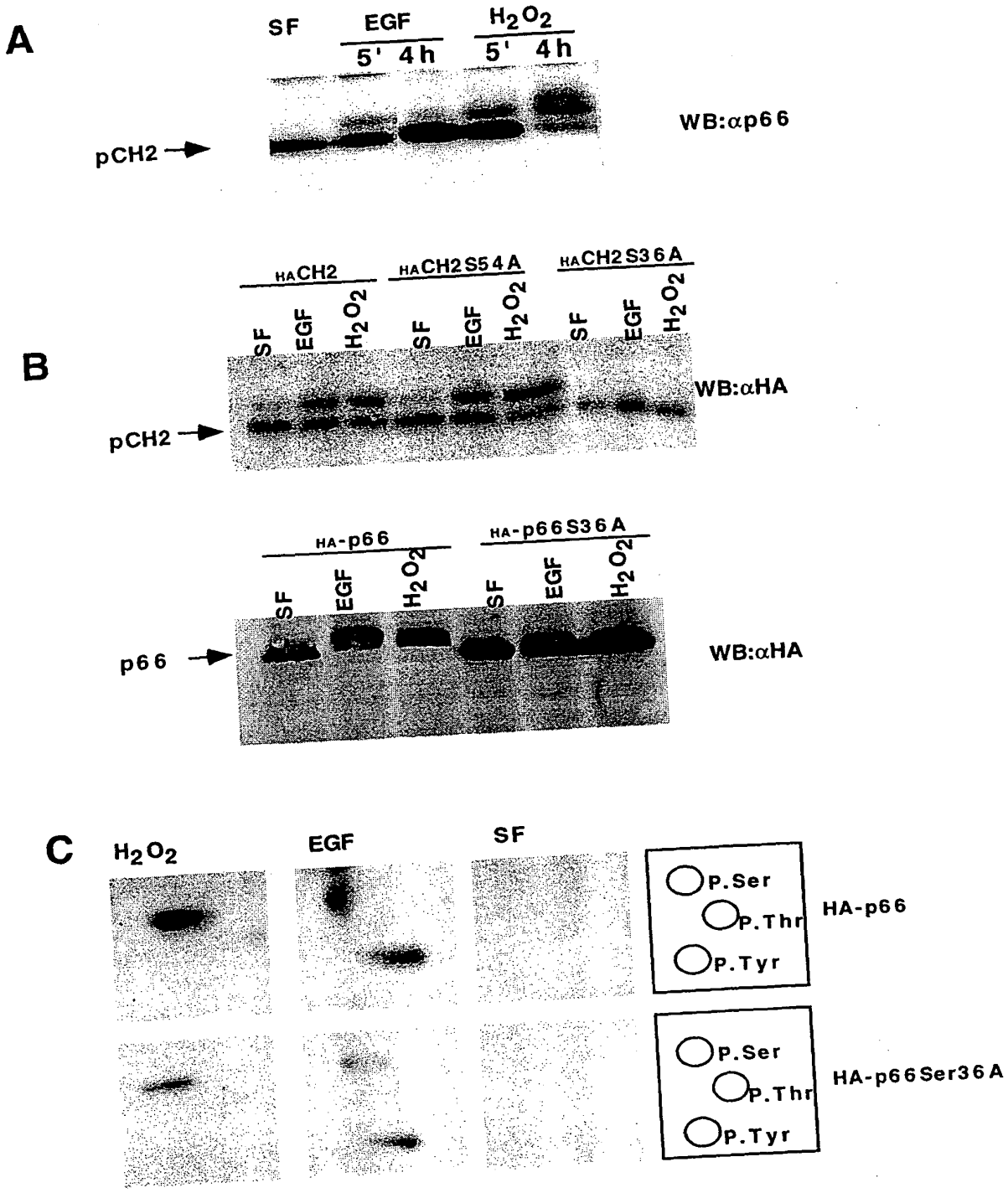

A
SF
EGF
5' 4h
H2O2
5' 4h
pCH2
WB:αp66
B
HACH2
HACH2S54A
HACH2S36A
SF
EGF
H2O2
pCH2
WB:αHA
HA-p66
HA-p66S36A
p66
WB:αHA
C
H2O2
EGF
SF
P.Ser
P.Thr
P.Tyr
HA-p66
HA-p66Ser36A

Fig. 3

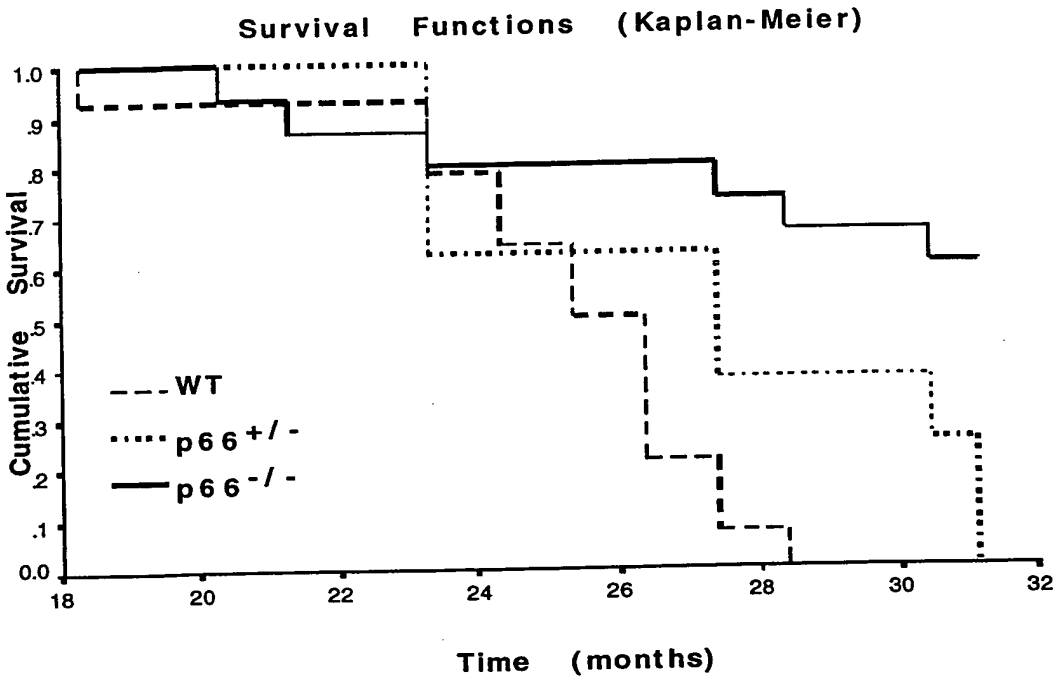

Survival Functions (Kaplan-Meier)
Cumulative Survival
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0.0
18
20
22
24
26
28
30
32
WT
p66+/-
p66-/-
Time (months)

Fig. 4

66 cDNA nucleotide [redacted].

```
        10         20         30         40         50         60
atggggcctg aaactgtctg ggtctgagct ggggagcgga agccacttgt ccctctccct

        70         80         90        100        110        120
ccccaggact tctgtgactc ctgggccaca gaggtccaac cagggtaagg gcctggggat

       130        140        150        160        170        180
acccccctgcc tggccccctt gcccaaactg gcaggggggc caggctgggc agcagccccct

       190        200        210        220        230        240
ctttcacctc aactATGGAT CTCCTGCCCC CCAAGCCCAA GTACAATCCA CTCCGGAATG

       250        260        270        280        290        300
AGTCTCTGTC ATCGCTGGAG GAAGGGGCTT CTGGGTCCAC CCCCCCGGAG GAGCTGCCTT

       310        320        330        340        350        360
CCCCATCAGC TTCATCCCTG GGGCCCATCC TGCCTCCTCT GCCTGGGGAC GATAGTCCCA

       370        380        390        400        410        420
CTACCCTGTG CTCCTTCTTC CCCCGGATGA GCAACCTGAG GCTGGCCAAC CCGGCTGGGG

       430        440        450        460        470        480
GGCGCCCAGG GTCTAAGGGG GAGCCAGGAA GGGCAGCTGA TGATGGGGAG GGGATCGATG

       490        500        510        520        530        540
GGGCAGCCAT GCCAGAGTCA GGCCCCCTAC CCCTCCTCCA GGACATGAAC AAGCTGAGTG

       550        560        570        580        590        600
GAGGCGGCGG GCGCAGGACT CGGGTGGAAG GGGGCCAGCT TGGGGGCGAG GAGTGGACCC

       610        620        630        640        650        660
GCCACGGGAG CTTTGTCAAT AAGCCCACGC GGGGCTGGCT GCATCCCAAC GACAAAGTCA

       670        680        690        700        710        720
TGGGACCCGG GGTTTCCTAC TTGGTTCGGT ACATGGGTTG TGTGGAGGTC CTCCAGTCAA

       730        740        750        760        770        780
TGCGTGCCCT GGACTTCAAC ACCCGGACTC AGGTCACCAG GGAGGCCATC AGTCTGGTGT

       790        800        810        820        830        840
GTGAGGCTGT GCCGGGTGCT AAGGGGGCGA CAAGGAGGAG AAAGCCCTGT AGCCGCCCGC

       850        860        870        880        890        900
TCAGCTCTAT CCTGGGGAGG AGTAACCTGA AATTTGCTGG AATGCCAATC ACTCTCACCG

       910        920        930        940        950        960
TCTCCACCAG CAGCCTCAAC CTCATGGCCG CAGACTGCAA ACAGATCATC GCCAACCACC

       970        980        990       1000       1010       1020
ACATGCAATC TATCTCATTT GCATCCGGCG GGGATCCGGA CACAGCCGAG TATGTCGCCT

      1030       1040       1050       1060       1070       1080
ATGTTGCCAA AGACCCTGTG AATCAGAGAG CCTGCCACAT TCTGGAGTGT CCCGAAGGGC

      1090       1100       1110       1120       1130       1140
TTGCCCAGGA TGTCATCAGC ACCATTGGCC AGGCCTTCGA GTTGCGCTTC AAACAATACC

      1150       1160       1170       1180       1190       1200
TCAGGAACCC ACCCAAACTG GTCACCCCTC ATGACAGGAT GGCTGGCTTT GATGGCTCAG

      1210       1220       1230       1240       1250       1260
CATGGGATGA GGAGGAGGAA GAGCCACCTG ACCATCAGTA CTATAATGAC TTCCCGGGGA

      1270       1280       1290       1300       1310       1320
AGGAACCCCC CTTGGGGGGG GTGGTAGACA TGAGGCTTCG GGAAGGAGCC GCTCCAGGGG

      1330       1340       1350       1360       1370       1380
CTGCTCGACC CACTGCACCC AATGCCCAGA CCCCCAGCCA CTTGGGAGCT ACATTGCCTG
```

Fig. 5

56 cDNA nucleotide [redacted].

```
      1390        1400        1410        1420        1430        1440
TAGGACAGCC TGTTGGGGGA GATCCAGAAG TCCGCAAACA GATGCCACCT CCACCACCCT

      1450        1460        1470        1480        1490        1500
GTCCAGGCAG AGAGCTTTTT GATGATCCCT CCTATGTCAA CGTCCAGAAC CTAGACAAGG

      1510        1520        1530        1540        1550        1560
CCCGGCAAGC AGTGGGTGGT GCTGGGCCCC CCAATCCTGC TATCAATGGC AGTGCACCCC

      1570        1580        1590        1600        1610        1620
GGGACCTGTT TGACATGAAG CCCTTCGAAG ATGCTCTTCG GGTGCCTCCA CCTCCCCAGT

      1630        1640        1650        1660        1670        1680
CGGTGTCCAT GGCTGAGCAG CTCCGAGGGG AGCCCTGGTT CCATGGGAAG CTGAGCCGGC

      1690        1700        1710        1720        1730        1740
GGGAGGCTGA GGCACTGCTG CAGCTCAATG GGGACTTCTT GGTACGGGAG AGCACGACCA

      1750        1760        1770        1780        1790        1800
CACCTGGCCA GTATGTGCTC ACTGGCTTGC AGAGTGGGCA GCCTAAGCAT TTGCTACTGG

      1810        1820        1830        1840        1850        1860
TGGACCCTGA GGGTGTGGTT CGGACTAAGG ATCACCGCTT TGAAAGTGTC AGTCACCTTA

      1870        1880        1890        1900        1910        1920
TCAGCTACCA CATGGACAAT CACTTGCCCA TCATCTCTGC GGGCAGCGAA CTGTGTCTAC

      1930        1940        1950        1960        1970        1980
AGCAACCTGT GGAGCGGAAA CTGtgatctg ccctagcgct ctcttccaga agatgccctc

      1990        2000        2010        2020        2030        2040
caatcctttc caccctattc cctaactctc gggacctcgt ttgggagtgt tctgtgggct

      2050        2060        2070        2080        2090        2100
tggccttgtg tcagagctgg gagtagcatg gactctgggt ttcatatcca gctgagtgag

      2110        2120        2130        2140        2150        2160
agggtttgag tcaaaagcct gggtgagaat cctgcctctc cccaaacatt aatcaccaaa

      2170        2180        2190        2200        2210        2220
gtattaatgt acagagtggc ccctcacctg ggcctttcct gtgccaacct gatgcccctt

      2230        2240        2250        2260        2270        2280
ccccaagaag gtgagtgctt gtcatggaaa atgtcctgtg gtgacaggcc cagtggaaca

      2290        2300        2310        2320        2330        2340
gtcaccttc tgggcaaggg ggaacaaatc acacctctgg gcttcagggt atcccagacc

      2350        2360        2370        2380        2390        2400
cctctcaaca cccgcccccc ccatgtttaa actttgtgcc tttgaccatc tcttaggtct

      2410        2420        2430        2440        2450        2460
aatgatattt tatgcaaaca gttcttggac ccctgaattc ttcaatgaca gggatgccaa

      2470        2480        2490        2500        2510        2520
caccttcttg gcttctggga cctgtgttct tgctgagcac cctctccggt ttgggttggg

      2530        2540        2550        2560        2570        2580
ataacagagg caggagtggc agctgtcccc tctccctggg gatatgcaac ccttagagat

      2590        2600        2610        2620        2630        2640
tgccccagag ccccactccc ggccaggcgg gagatggacc cctcccttgc tcagtgcctc

      2650        2660        2670        2680        2690        2700
ctggccgggg ccccctcaccc caaggggtct gtatatacat ttcataaggc ctgccctccc

      2710        2720        2730        2740        2750        2760
```

p[redacted] cDNA nucleotide [redacted]q.

atgttgcatg cctatgtact ctgcgccaaa gtgcagccct tcctcctgaa gcctctgccc

2770 2780 2790 2800 2810 2820
tgcctccctt tctgggaggg cggggtgggg gtgactgaat ttgggcctct tgtacagtta

2830 2840 2850 2860 2870 2880
actctcccag gtggattttg tggaggtgag aaaaggggca ttgagactat aaagcagtag

2890 2900 2910 2920 2930 2940
acaatcccca cataccatct gtagagttgg aactgcattc ttttaaagtt ttatatgcat

2950 2960 2970 2980 2990 3000
atattttagg gctgctagac ttactttcct attttctttt ccattgctta ttcttgagca

3010 3020 3030 3040 3050 3060
caaaatgata atcaattatt acatttatac atcacctttt tgacttttcc aagccctttt

3070 3080 3090 3100 3110 3120
acagctcttg gcattttcct cgcctaggcc tgtgaggtaa ctgggatcgc acctttttata

3130 3140 3150 3160 3170 3180
ccagagacct gaggcagatg aaatttattt ccatctagga ctagaaaaac ttgggtctct

3190 3200 3210 3220 3230 3240
taccgcgaga ctgagaggca gaagtcagcc cgaatgcctg tcagtttcat ggaggggaaa

3250 3260 3270 3280 3290 3300
cgcaaaacct gcagttcctg agtaccttct acaggcccgg cccagcctag gcccggggtg

3310 3320 3330 3340 3350 3360
gccacaccac agcaagccgg ccccccctct tttggccttg tggataaggg agagttgacc

3370 3380 3390 3400 3410 3420
gttttcatcc tggcctcctt ttgctgtttg gatgtttcca cgggtctcac ttataccaaa

3430 3440 3450 3460 3470 3480
gggaaaactc ttcattaaag tccgtatttc ttctaaaaaa aaaaaaaaaa aaatacattt

3490 3500 3510 3520 3530 3540
atacatcacc tttttgactt ttccaagccc ttttacagct cttggcattt tcctcgccta

3550 3560 3570 3580 3590 3600
ggcctgtgag gtaactggga tcgcaccttt tataccagag acctgaggca gatgaaattt

3610 3620 3630 3640 3650 3660
atttccatct aggactagaa aaacttgggt ctcttaccgc gagactgaga ggcagaagtc

3670 3680 3690 3700 3710 3720
agcc..... ....

8/11

p66 aa sequence

```
        10         20         30         40         50         60
MDLLPPKPKY NPLRNESLSS LEEGASGSTP PEELPSPSAS SLGPILPPLP GDDSPTTLCS

        70         80         90        100        110        120
FFPRMSNLRL ANPAGGRPGS KGEPGRAADD GEGIDGAAMP ESGPLPLLQD MNKLSGGGGR

       130        140        150        160        170        180
RTRVEGGQLG GEEWTRHGSF VNKPTRGWLH PNDKVMGPGV SYLVRYMGCV EVLQSMRALD

       190        200        210        220        230        240
FNTRTQVTRE AISLVCEAVP GAKGATRRRK PCSRPLSSIL GRSNLKFAGM PITLTVSTSS

       250        260        270        280        290        300
LNLMAADCKQ IIANHHMQSI SFASGGDPDT AEYVAYVAKD PVNQRACHIL ECPEGLAQDV

       310        320        330        340        350        360
ISTIGQAFEL RFKQYLRNPP KLVTPHDRMA GFDGSAWDEE EEEPPDHQYY NDFPGKEPPL

       370        380        390        400        410        420
GGVVDMRLRE GAAPGAARPT APNAQTPSHL GATLPVGQPV GGDPEVRKQM PPPPPCPGRE

       430        440        450        460        470        480
LFDDPSYVNV QNLDKARQAV GGAGPPNPAI NGSAPRDLFD MKPFEDALRV PPPPQSVSMA

       490        500        510        520        530        540
EQLRGEPWFH GKLSRREAEA LLQLNGDFLV RESTTTPGQY VLTGLQSGQP KHLLLVDPEG

       550        560        570        580
VVRTKDHRFE SVSHLISYHM DNHLPIISAG SELCLQQPVE RKL*
```

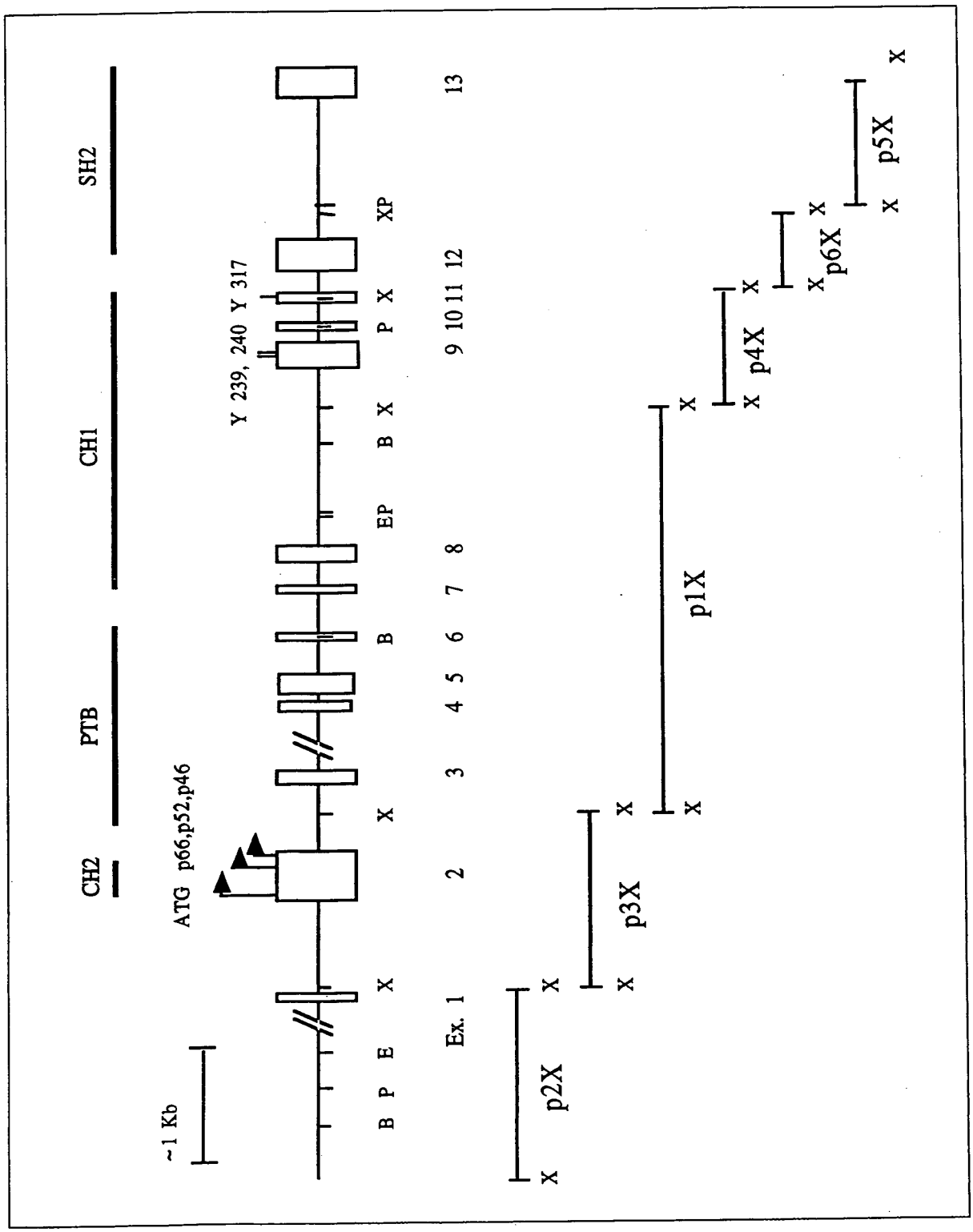

SH2
CH1
PTB
CH2
ATG p66,p52,p46
~1 Kb
B P E X
Ex. 1 2 3 4 5 6 7 8 9 10 11 12 13
X B EP B X P X XP
Y 239, 240 Y 317
p2X
p3X
p1X
p4X
p6X
p5X

Fig. 6

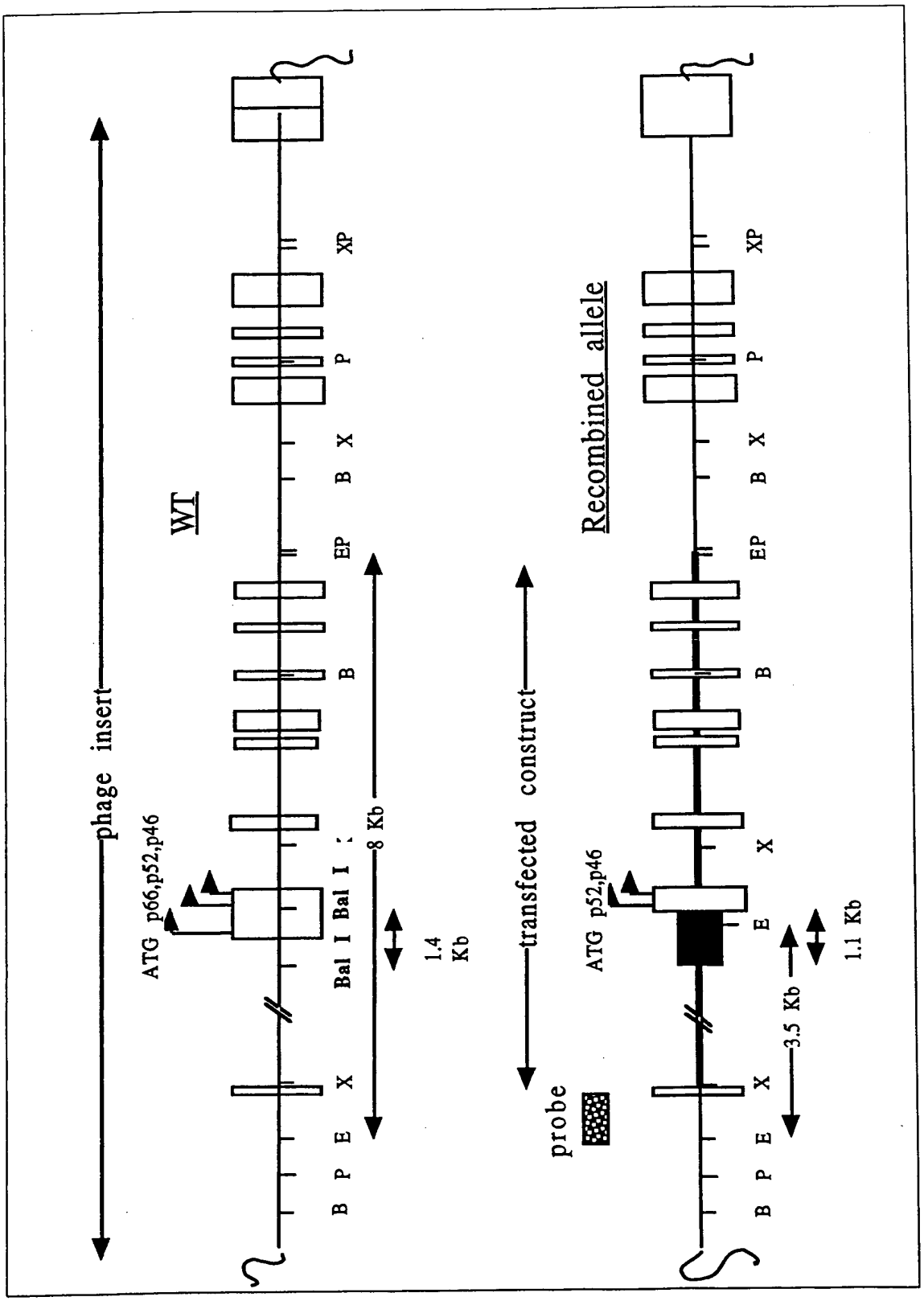

phage insert
WT
ATG p66,p52,p46
B P E X Bal I Bal I B EP B X P XP
8 Kb
1.4 Kb
transfected construct
probe
Recombined allele
ATG p52,p46
B P E X 3.5 Kb
E X B EP B X P XP
1.1 Kb

Fig. 7

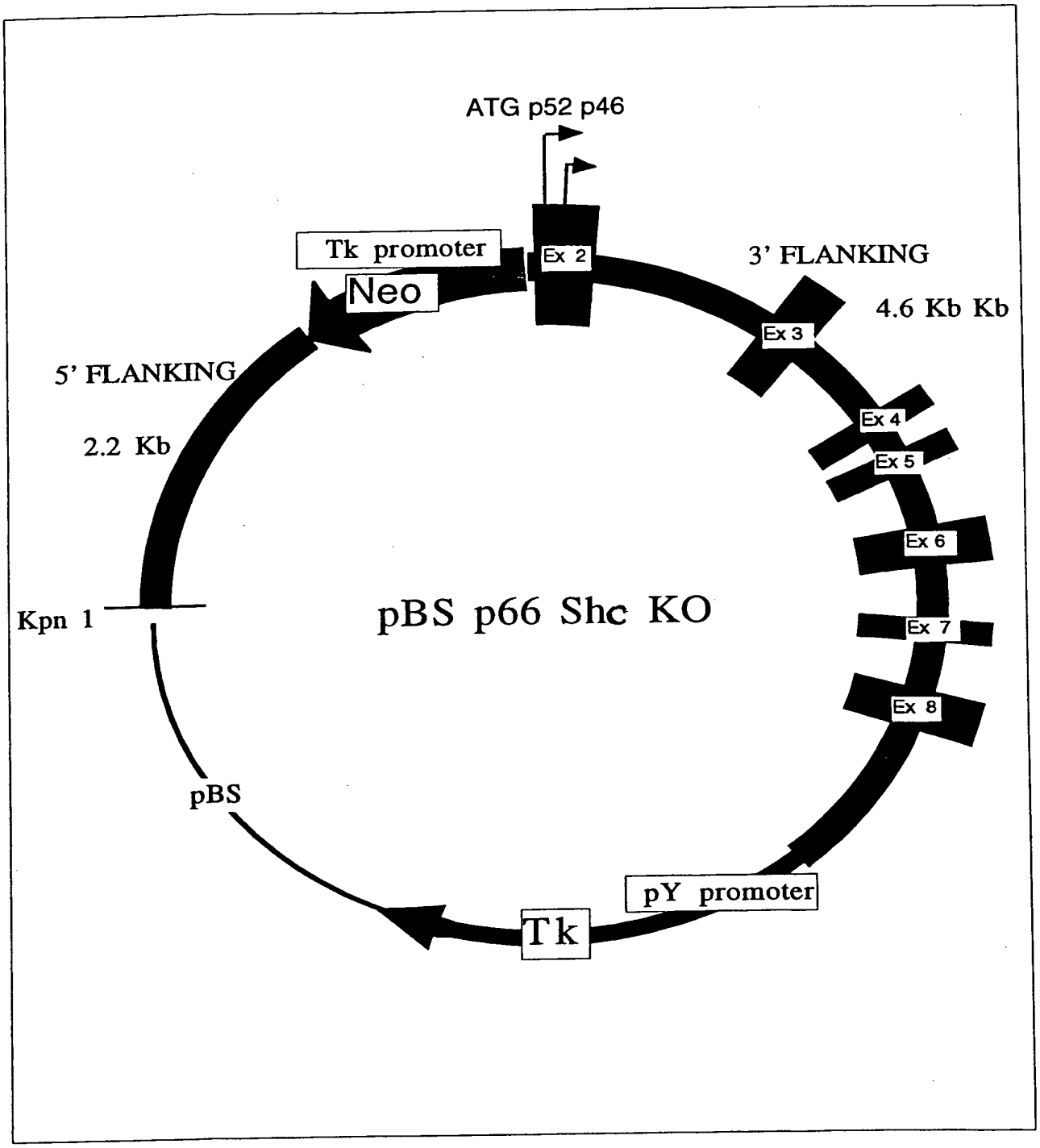

ATG p52 p46
Tk promoter
Ex 2
3' FLANKING
4.6 Kb Kb
Neo
Ex 3
5' FLANKING
2.2 Kb
Ex 4
Ex 5
Ex 6
Kpn 1
pBS p66 Shc KO
Ex 7
Ex 8
pBS
pY promoter
Tk

Fig. 8

LOT NO : G800 / 1079

Form 23/77 : 22.3.00

Agent : Newbury Ellis